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Microbial succession in a compost-packed biofilter treating benzene-contaminated air

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Abstract

Air artificially contaminated with increasing concentrations of benzene was treated in a laboratory scale compost-packed biofilter for 240 days with a removal efficiency of 81–100%. The bacterial community in the packing material (PM) at different heights of the biofilter was analysed every 60 days. Bacterial plate counts and ribosomal intergenic spacer analysis (RISA) of the isolated strains showed that the number of cultivable aerobic heterotrophic bacteria and the species diversity increased with benzene availability. Identification of the isolated species and the main bands in denaturing gradient gel electrophoresis (DGGE) profiles from total compost DNA during the treatment revealed that, at a relatively low volumetric benzene load ($1.2 \leq VBL \leq 6.4$ g m^{-3}_{PM} h^{-1}), besides low G+C Gram positive bacteria, originally present in the packing compost, bacteroidetes and β- and γ-proteobacteria became detectable in the colonising population. At the VBL value (24.8 g m^{-3}_{PM} h^{-1}) ensuring the maximum elimination capacity of the biofilter (20.1 g m^{-3}_{PM} h^{-1}), strains affiliated to the genus *Rhodococcus* dominated the microflora, followed by β-proteobacteria comprising the genera *Bordetella* and *Neisseria*. Under these conditions, more than 35% of the isolated strains were able to grow on benzene as the sole carbon source. Comparison of DGGE and automated RISA profiles of the total community and isolated strains showed that a complex bacterial succession occurred in the reactor in response to the increasing concentrations of the pollutant and that cultivable bacteria played a major role in benzene degradation under the adopted conditions.

Introduction

Biofiltration provides an effective and inexpensive technology for the removal of volatile, toxic or odorous compounds which contaminate air emissions from industrial plants treating petroleum components, wastes, food or animal-rendering, or for the removal of toxic compounds stripped from contaminated soils during *in situ* bioremediation (Leson & Winer 1991; Zilli & Converti 1999; Van Hamme et al. 2003).

Microorganisms grow as a biofilm on packed organic material flushed by the contaminated air, and oxidise the pollutants (Møller et al. 1996; Acuña et al. 1999). The packing medium sorbs contaminants from the vapour stream and may supply organic and/or inorganic compounds for microbial growth and metabolism, immobilises the microbial cells preventing washout, constitutes a nutritious and humid reservoir as well as a mechanical odourless support, minimises the overall reactor volume, energy consumption, and ensures high removal yields (Zilli et al. 2004).

Although biofiltration technologies have been well established and optimised for the remediation of various toxic compounds from air emissions

(Veiga & Kennes 2001; Sene et al. 2002; Van Hamme et al. 2003), microbiology of biofiltration has been investigated only in the past two decades. Several authors studied the cultivable fraction of the microflora, by identification and chemo-taxonomical description of heterotrophic or degrading strains isolated from the biofilter packing material (Ahrens et al. 1997; Lipski & Altendorf 1997; Juteau et al. 1999; Roy et al. 2003) and described their degrading potential (Hanson et al. 1999).

Recent studies applied molecular culture-independent methods to describe the composition of the microbial community during biofiltration (Friedrich et al. 2002) and to monitor the biofilter colonisation by specific degradative populations (Møller et al. 1996; Sakano & Kerkhof 1998).

Population fingerprinting methods, based on the amplification of portions of the ribosomal operon, allow to describe the structure of microbial communities. Moreover, the sequencing of PCR fragments in the fingerprinting profiles can lead to the identification of the microbial species present in the community giving insight on their diversity (Heuer & Smalla 1997). The isolation and characterisation of strains isolated from biofilters with efficient degrading capabilities would complement cultivation-independent methods leading to the unambiguous identification of the bacteria potentially active in the biodegradation, which could be used as inocula to shorten the start-up or acclimatation period of similar processes (Acuña et al. 1999; Veiga & Kennes 2001; Van Hamme et al. 2003). All together these methods can give a general picture of the microbial succession occurring in a biofilter in response to different operative conditions, hence giving information on how microbial communities adapt to changes of pollutant organic load.

In the present work we used a polyphasic approach for the study of the microbial ecology of a laboratory-scale compost biofilter, aiming to evaluate the succession of microbial species under different conditions of biofiltration. By using cultivation-dependent and independent approaches we studied the changes of the structure and diversity of the microbial community in a biofilter treating air polluted with benzene, a highly toxic volatile compound included in the polluted priority lists of most environmental protection agencies.

Materials and methods

Biofiltration process

A laboratory-scale biofilter consisted of a glass column with 0.05 m internal diameter, 0.65 m total height and 0.51 m packing material height, provided with sampling ports installed, along both sides, at 0.125, 0.250, 0.375, and 0.510 m from the bottom of the packing material. The column was packed with a mixture of powdered compost from the organic fraction of municipal solid waste and glass beads (5 mm-diameter) in a 4:1 volume proportion. The bioreactor was fluxed in upflow direction with air moisturised and artificially contaminated with benzene. A mineral salts solution (50 ml), flowing counter currently with the gas flowing upwards through the column, was monthly distributed at the top of the packing material and daily re-circulated to avoid possible depletion of nutrients in the compost. The composition of this solution was per litre of distilled water: 5 g Na_2HPO_4, 4 g KH_2PO_4, 4 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4{\cdot}7\ H_2O$, 0.34 g $MgCl_2{\cdot}6\ H_2O$, 0.08 g $FeSO_4{\cdot}7\ H_2O$, 0.07 g $CaCl_2{\cdot}6\ H_2O$, 0.002 g $ZnSO_4{\cdot}7\ H_2O$, and 0.002 g $MnSO_4{\cdot}H_2O$.

To evaluate the biofilter removal efficiency, continuous experiments were carried out under non sterile conditions at different benzene concentrations in the influent air stream, sequentially stepped up through three different superficial gas velocities, specifically 31, 61, and 122 m h^{-1}, yielding apparent gas residence times (τ) of 60, 30, and 15 s, respectively. The benzene removal efficiency was measured by gas-chromatography as described elsewhere (Sene et al. 2002).

The microbiological study was done, over a period of 240 days, on samples taken from the bed under the following operating conditions: concentration of benzene in the upflowing air (C_{go}) = 0.01, 0.02, 0.05 and 0.2 g m^{-3}, superficial gas velocity (U_g) = 122 m h^{-1}, corresponding to volumetric benzene loads (VBL) of 1.2, 2.4, 6.2 and 24.8 g $m^{-3}{}_{PM}\ h^{-1}$, respectively.

As the total porosity of the compost was 69.0%, the corresponding EBRT (Empty Bed Residence Time) was: 40, 20, and 10 s. To prevent shock and to acclimatize the indigenous microflora, the lowest inlet benzene concentration (C_{go} = 0.01 g m^{-3}) and

superficial gas velocity ($U_g = 31$ m h^{-1}) were used during the biofilter start-up that lasted about 20 days. Afterwards, U_g was progressively increased to 61 and then to 122 m h^{-1}, whereas the inlet benzene concentration was kept constant. When the first cycle was complete for one concentration, C_{go} was increased and the cycle was started again at the lowest U_g and following the same experimental schedule as that of the first cycle of tests.

Each cycle of assays, under a given set of operating conditions, lasted about 15 days, during which pseudo-steady-state conditions were usually achieved within about 2–3 days.

Analysis of cultivable microflora

The compost was sampled either before packing the column or at different heights of the column ($h = 0.125$, 0.250, 0.375, 0.510 m from the bottom) after 60, 120, 180 and 240 days of treatment.

For the total microbial counts 100 μl of serial dilutions of 0.5 g of compost in sterile saline solution (NaCl 9 g l^{-1}) were plated in triplicate on Plate Count Agar (Merk, Milano, Italy) supplemented with cycloheximide (100 μg/ml in water) (Sigma-Aldrich, Milano, Italy) to prevent fungal growth and incubated 3 days at 30 °C before the count of the colonies. Isolated colonies were subcultured in the same medium.

Bacterial DNA was extracted with the method of Ausubel et al. (1994) and RISA analysis was performed as described by Daffonchio et al. (1998). Selected strains were identified by partial sequencing of the first 300–500 bp of the 16S rRNA gene as described in Urzì et al. (2001). Strain diversity of isolates belonging to the same species has been estimated by Rep-PCR-fingerprinting as described by Cherif et al. (2003).

Strains identified as *Neisseria* have been tested by specific PCR assays. *Neisseria*-specific primers were asd21 and asd102, while *N. meningitidis*-specific oligonucleotide primers were omp56 and omp212 (Lansac et al. 2000). Each reaction mixture of 25 μl contained 0.3 μM of primers, 0.12 mM dNTPs (Amersham, Milano, Italy) and 0.5 U of *Taq*-polymerase with the provided 1 × buffer (Amersham). The PCR mixtures were subjected to thermal cycling (3 min at 94 °C and 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, with a final extension at 72 °C for 4 min) in an I-cycler thermal cycler (Bio-Rad Milano, Italy). Five μl of each PCR product were resolved by electrophoresis in a 2% agarose gel in Tris–borate–EDTA buffer and visualised after ethidium bromide staining of the gel, under ultraviolet light.

Pure cultures isolated at the 240th day of treatment were streaked on M9 medium (Sambrook et al. 1989) agarised with noble agar (Difco, Milano, Italy). Inoculated plates were incubated for 10 days at 30 °C in closed jars either in the presence or the absence of benzene vapours. Strains exhibiting clear growth only in the presence of benzene were scored as positive for benzene utilisation.

DNA-fingerprinting analysis of total microflora

Total compost DNA was extracted with a commercial kit (Qbiogene, Carlsbad, USA) from 0.5 g of material stored at −20 °C after the sampling, following the instructions of the manufacturer.

ARISA fingerprint was performed on the extracted DNA as described elsewhere (Brusetti et al. 2004). DGGE fingerprint was performed as described by Sass et al. (2001) with the primers GC357f and 907r, separating the PCR amplicons in a 7% polyacrylamide gel with a denaturing gradient of urea and formamide of 40% (top) – 60% (bottom), where 100% denaturation is considered urea 7 M and formamide 40%. The electrophoresis was run at 110 V for 14 h at 58 °C in a D-Code apparatus (Bio-Rad). The gel was stained in a solution 1× of SybrGreen (Molecular Probes, Leiden, The Netherlands) for 30 min and its image captured in UV transillumination with a digital camera supported by a Gel Doc 2000 apparatus (Bio-Rad). Bands of interest were cut from the gel with a sterile scalpel; the DNA was extracted by incubating the gel fragments for 12 h in 100 μl of sterile distilled water at 37 °C under agitation; 10 μl of the solution were then used as template to re-amplify the fragment using the same DGGE primers without the GC-clamp and the same PCR conditions applied to the original compost DNA. The obtained amplicons were then sequenced with the same protocol adopted for

the pure strains identification, using the primer 907r.

Neisseria-specific primers asd21 and asd102 were tested on the total compost DNA with the same PCR protocol applied for the pure strains.

Results

Biofilter performance

The main results of biofilter performance obtained under the different operative conditions tested throughout this study had already been presented and discussed in detail (Zilli et al. 2005). In particular, the benzene removal was shown to be almost complete under milder conditions ($C_{go} \leq 0.20$ g m^{-3} at $U_g = 31$ m h^{-1}; $C_{go} \leq 0.10$ g m^{-3} at $U_g = 61$ m h^{-1}; $C_{go} \leq 0.020$ g m^{-3} at $U_g = 122$ m h^{-1}). Benzene elimination capacity increased regularly with volumetric benzene load (VBL) up to a maximum of 20.1 g m$^{-3}_{PM}$ h^{-1} (at VBL = 24.8 g m$^{-3}_{PM}$ h^{-1}, corresponding to $\eta = 0.81$) over which it decreased due to excess benzene inhibition (Zilli et al. 2005). The biofilter performance markedly improved when progressively increasing C_{go} at a given VBL, likely due to a rise in the diffusion driving force. Analogously, a decrease in U_g may have increased the time of benzene diffusion. Both phenomena could have been responsible, on the whole, for the different VBL thresholds above which the efficiency decreased (Zilli et al. 2005).

Summarizing, the biofilter showed a benzene removal efficiency close to 100% for the first 120 days of operation when the VBL was progressively increased from 1.2 to 2.4 g m$^{-3}_{PM}$ h^{-1} and then decreased to 88% at 6.2 g m$^{-3}_{PM}$ h^{-1}. During the last 20 days, operating at the highest values of U_g (122 m h^{-1}) and C_{go} (0.2 g m^{-3}), corresponding to VBL = 24.8 g m$^{-3}_{PM}$ h^{-1}, the biofilter still behaved very effectively showing a good removal efficiency of 81%, corresponding to a maximum benzene elimination capacity of 20.1 g m$^{-3}_{PM}$ h^{-1}.

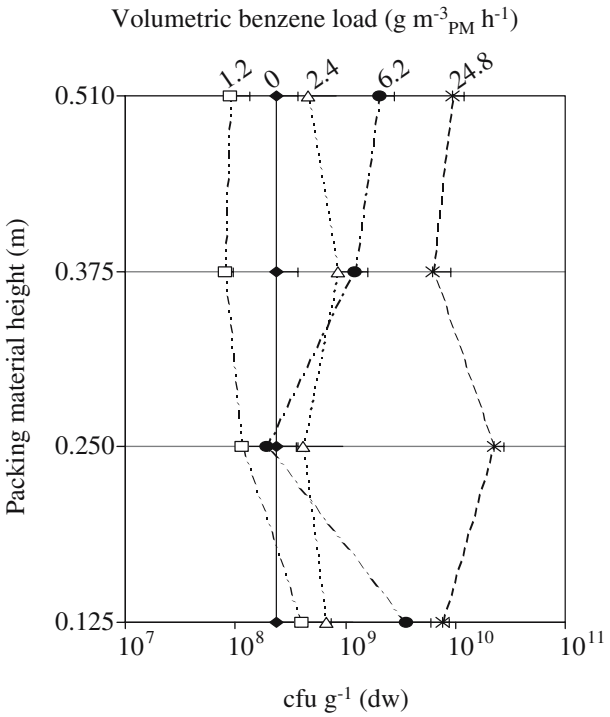

Figure 1. Bacterial plate counts in the original compost before the column packing (0) and from compost samples taken at different heights from the bottom of the packing material, operating at VBL = 1.2, 2.4, 6.2 and 24.8 g m$^{-3}_{PM}$ h^{-1}.

Analysis of cultivable microflora during 240 days of biofiltration

After exposure to benzene at VBL = 1.2 g m^{-3}_{PM} h^{-1}, the bacterial number slightly decreased at almost all the heights of the packing material (Figure 1) if compared with the original compost (2.8 10^8 cfu g^{-1}_{dw}) and tended to increase in the following two periods of biofiltration (VBL = 2.4 and 6.2 g m^{-3}_{PM} h^{-1}). During the last biofiltration phase, i.e. between 180 and 240 days of operation, in parallel with a strong increase in VBL (up to 24.8 g m^{-3}_{PM} h^{-1}) the microbial colonisation of the support showed a sharp increment, as demonstrated by the increased number of colonies (about 10^{10} cfu g^{-1}_{dw}) at all the heights of the biofilter.

From the Petri dishes used for plate counts which exhibited a number of separated colonies between 10 and 100, bacterial strains were isolated at each sampling time, establishing a collection of about 200 strains. Species diversity of the strains was analysed by RISA fingerprinting which yields species/subspecies-specific band profiles (Daffonchio et al. 1998). This analysis could divide the collection into groups of the same species/subspecies, that were then identified by partial sequencing of the 16S rRNA gene of one or two strains for the most abundant RISA groups. The species/subspecies variability increased along the treatment: in the original compost we were able to identify only three RISA groups among the isolates, while the diversity increased to eight, six and eleven different RISA groups after exposure to increasing VBL (VBL of 1.2, 2.4 and 6.2 g m^{-3}_{PM} h^{-1} respectively). The number of RISA groups almost duplicated in the last period reaching 21 detectable groups at VBL = 2.4 g m^{-3}_{PM} h^{-1}, consistently with the increased organic load and microbial counts. In the unpacked compost we identified species typically isolated from this material, such as actinobacteria and aerobic sporeformers (Peters et al. 2000). During the process new cultivable species appeared over the detection limit of the method, belonging to other phylogenetic groups including β- and γ-proteobacteria and high G + C Gram positives (Table 1). The diversity in the taxonomic groups which yielded the highest number of strains at VBL = 24.8 g m^{-3}_{PM} h^{-1}, i.e. *Rhodococcus*, *Neisseria* and *Pseudoxanthomonas*, was analysed by typing the strains by rep-PCR (Cherif et al. 2003). In all the three groups we found the presence of more than one strain (Table 1).

Table 1. Species identification of the main RISA groups isolated from the biofilter, Rep-PCR strain characterisation and growth on benzene as the sole carbon source

RISA groups n°[a]	Strains n°	Rep-PCR haplotypes n°	rRNA gene Sequence affiliation	%[b]	Phylogenetic group	VBLs of isolation (g m^{-3}_{PM} h^{-1})	Benzene[c]
1	9	n.d.	*Agromyces*	91	Actinobacteria	0, 6.2, 24.8	3
2	19	n.d.	*Bacillus cereus*	96	Firmicutes-bacillales	0, 1.2, 2.4, 6.2	0
1	12	n.d.	*Bacillus thuringiensis*	99	Firmicutes-bacillales	1.2, 6.2, 24.8	1
1	20	n.d.	*Bacillus subtilis*	96	Firmicutes-bacillales	1.2, 2.4, 24.8	0
1	1	n.d.	*Staphylococcus*	97	Firmicutes-bacillales	24.8	0
1	1	n.d.	*Streptococcus mitis*	93	Firmicutes-lactobacillales	24.8	0
1	1	n.d.	*Zoogloea ramigera*	95	β-proteobacteria	24.8	0
1	10	n.d.	*Alcaligenes faecalis*	98	β-proteobacteria	24.8	0
2	7	n.d.	*Bordetella petrii*	98	β-proteobacteria	24.8	5
2	20	3	*Neisseria*	97	β-proteobacteria	24.8	2
2	11	n.d.	*Variovorax*	98	β-proteobacteria	24.8	0
1	5	n.d.	*Xenophilus azovorans*	98	β-proteobacteria	24.8	0
3	25	4	*Pseudoxanthomonas*	97	γ-proteobacteria	24.8	8
3	62	4	*Rhodococcus opacus*	96	Actinobacteria	24.8	58

[a] Number of RISA groups with the same identification record, based on rRNA gene sequence affiliation.
[b] Percent of homology reported as first score by Blast alignment of the sequences.
[c] Number of strains which showed growth on M9 medium supplemented with benzene as the sole carbon source.
n.d.: Not determined.

As reported for other compost-based biofilters (Juteau et al. 1999), the packing material was shown to be mainly colonised by actinomycetes and β-proteobacteria at the end of the process. Besides, we identified several strains of the genus *Neisseria*, which is known to be a primarily commensal of the mucous membranes of mammals and was never isolated in environmental matrices. The identification of these strains as *Neisseria* sp. was confirmed by the PCR amplification of the *asd* gene which is specific for such a genus (Lansac et al. 2000) (Figure 2). On the basis of the *ctr*A gene specific for the species *N. meningitidis* (Lansac et al. 2000) which did not yield the expected amplicon, it can be concluded that the strains did not belong to this species, but rather to other strains phylogenetically related to the pathogen (data not shown).

Benzene degradation

To collect information about the ecological significance of the bacteria isolated from the biofilter at the end of the process, we tested the strains isolated on the non selective medium Plate Count Agar under the best biofiltration conditions, i.e. under conditions (VBL = 24.8 g m^{-3}_{PM} h^{-1}) ensuring the maximum benzene elimination capacity (20.1 g m^{-3}_{PM} h^{-1}), when the microflora showed the largest abundance and diversity. Thirty-eight percent of the tested strains grew on benzene, and this capacity was distributed among different phylogenetic groups (Table 1). Moreover, it was mainly associated with members of the genus *Rhodococcus*, known to have degrading activities on aromatic compounds (Cavalca et al. 2000). Interestingly, some strains belonging to the genera *Neisseria* and *Bordetella*, known to include mammal commensal species never correlated with benzene degradation, positively grew on this compound.

Cultivation-independent analyses of bacterial community

The overall diversity of bacterial community was analysed by PCR-DGGE of 16s rRNA gene and ARISA fingerprinting and by PCR of signature genes specific for *Neisseria* (*Neisseria* genus-specific *asd*), performed on the total DNA directly extracted from the packing material.

Figure 3 shows the PCR-DGGE profiles originated from the compost at the different VBLs along the column height, while the identification of the bacteria originating the bands depicted in this figure is reported in Table 2. The PCR-DGGE and sequencing confirmed in part the results obtained for the cultivable microflora: the original compost was dominated by *Firmicutes* whose bands represent the main products in the PCR-DGGE profiles. During the biofiltration at increasing VBL other phylogenetic groups appeared, like α-, β- and γ-proteobacteria and actynomycetes. Intense bands were associated to *B. petrii*, *X. azovorans* and *Rhodococcus*, which appeared as dominant bands in the gel since VBL = 2.4 g m^{-3}_{PM} h^{-1} (*B. petrii* and *Rhodococcus*) or in the last biofiltration period at VBL = 24.8 g m^{-3}_{PM} h^{-1} (*X. azovorans*) and corresponded to some of the main strains isolated at VBL = 24.8 g m^{-3}_{PM} h^{-1}. Besides these, sequencing of the PCR-DGGE bands revealed the presence of other species undetected among the cultivated strains, like α-proteobacteria and species belonging to the CFB group, which were respectively undetectable or present in low amount in the original compost and

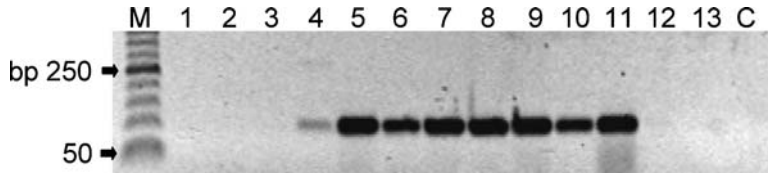

Figure 2. Results of the PCR amplification of the *Neisseria* genus-specific *asd* gene. Lane M: molecular ruler 50 bp ladder. Lanes 1–7: compost total DNA; 1: original compost; 2: VBL = 2.4 g m^{-3}_{PM} h^{-1} , h = 0.250 m; 3: VBL = 6.2 g m^{-3}_{PM} h^{-1}, h = 0.250 m; 4: VBL = 24.8 g m^{-3}_{PM} h^{-1}, h = 0.125 m; 5: VBL = 24.8 g m^{-3}_{PM} h^{-1}, h = 0.250 m; 6: VBL = 24.8 g m^{-3}_{PM} h^{-1}, h = 0.375 m; 7: VBL = 24.8 g m^{-3}_{PM} h^{-1}, h = 0.510 m. Lanes 8–11: pure strains identified as *Neisseria*, strains 213, 109, 50, 503. Lanes 12–13: pure strains of different species: *Pseudoxanthomonas* strain 110, *Rhodococcus* strain 302. C: PCR negative control without DNA template.

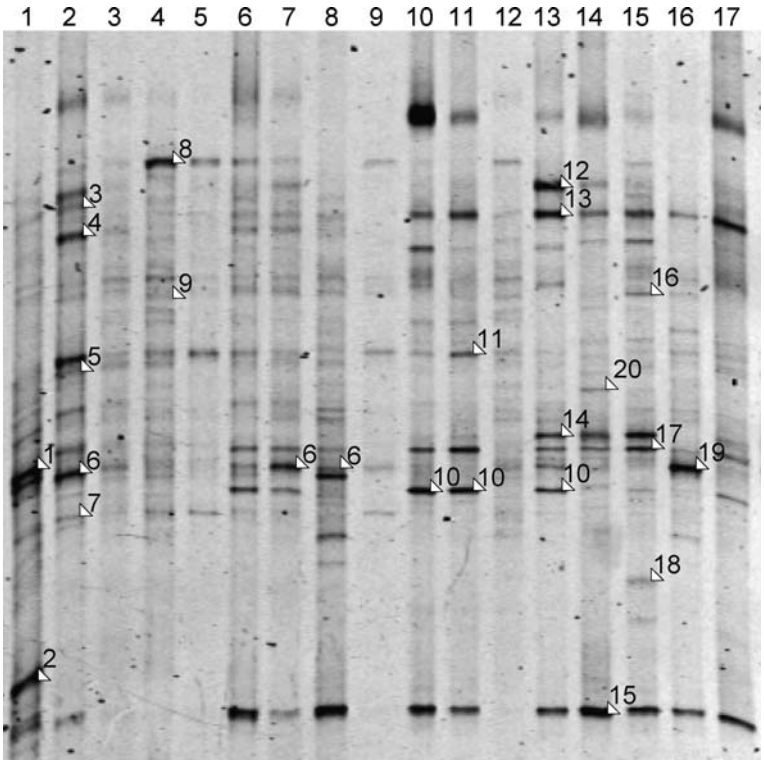

Figure 3. 16S rRNA gene DGGE profiles of the bacterial community of compost samples taken at different column heights and VBLs. Lane 1: original compost. Lanes: 2–5: VBL = 1.2 g m^{-3}_{PM} h^{-1}, h = 0.125, 0.250, 0.375, 0.510 m; Lanes 6–9: VBL = 2.4 g m^{-3}_{PM} h^{-1}, h = 0.125, 0.250, 0.375, 0.510 m; Lanes 10–13: VBL = 6.2 g m^{-3}_{PM} h^{-1}, h = 0.125, 0.250, 0.375, 0.510 m; Lanes: 14–17: VBL = 24.8 g m^{-3}_{PM} h^{-1}, h = 0.125, 0.250, 0.375, 0.510 m. Triangles indicate DNA fragments that were sequenced. Number refer to identified bands reported in Table 2.

were equally distributed along the column during the entire process.

ARISA profiles (Figure 4) showed that the biofiltration process selected a rich bacterial community clearly different from the one in the original compost, with the appearance of several peaks with length between 430 and 730 bp. By comparing the community ARISA profile of compost during the treatment with that of *Rhodococcus*, it was possible to identify its peak pattern, which was absent at the onset of the process, but appeared at VBL = 1.2 g m^{-3}_{PM} h^{-1} and whose intensity increased with VBL. As already observed in the PCR-DGGE patterns and by cultivation, the strongest peaks were those obtained at 24.8 g m^{-3}_{PM} h^{-1}.

Neisseria specific bands could not be observed in the PCR-DGGE and ARISA patterns, but the *Neisseria*-specific *asd*-gene was amplified from the total compost DNA when VBL was 24.8 g m^{-3}_{PM} h^{-1} as from the DNA of strains identified as *Neisseria* (Figure 2).

Discussion

In this work we described the microbial succession occurred during an efficient benzene biofiltration process utilising compost as packing material. The results obtained showed that the compost microflora sharply changed with increasing benzene organic load in the inlet air, so as to select for a complex microbial community rich in species and strain diversity. During the first period of operation, in which the microflora was exposed to low levels of benzene, a selection for strains with degrading potential occurred. These benzene-degrading bacteria were vehiculated by the ambient air or they were present in the original packing material under the limit of detection. During the

Table 2. Identification of the main bands in the DGGE patterns reported in Figure 3, and prevalence of the bands at different VBLs (g m^{-3}_{PM} h^{-1}) and heights from the bottom (*h*) of the biofilter

Band	Homology (GenBank)	Phylogenetic group	VBL	0	1.2				2.4				6.2				24.8			
			h		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	Unc. Bacterium	Firmicutes-bacillales		**x**	x															
18	Unc. Sludge bacterium																	**x**		
2	Unc. Bacterial clone	Firmicutes-clostridiales		**x**																
7	Unc. *citophagales*	Bacteroidetes		x	**x**		x	x	x			x				x				
3	Unc. CFB			x	**x**		x		x											
10	Unc. *bacteroidetes*												**x**	**x**		**x**		x		x
8	Unc. Bacterium				x		**x**	x	x	x		x	x		x			x		
9	Unc. *citophagales*				x		**x**	x												
12	*Cytophaga* sp.									x						**x**	x	x		
4	*Cytophaga* sp.				**x**	x	x		x	x	x									
13	*Cytophaga* sp.			x	**x**	x	x		x	**x**	**x**	x	x		x					
11	*Sphingomonas subartica*	α-proteobacteria			x	x	x	x	x	x	x	x	x	**x**	x	x	x	x	x	x
5	*Novosphingobium* sp.				**x**	x	x		x	x	x									
17	*Bordetella petrii*	β-proteobacteria							x	x	x		x	x		x	x	**x**	x	x
20	Gram negative bacterium																**x**			
19	*Xenophilus azovorans*														x	x	x	**x**	x	
6	Unc. Bacterium	γ-proteobacteria														**x**	x	x		x
14	*Luteimonas mephiti*								x	x	x		x	x	x	**x**	x	x	x	x
15	*Rhodococcus koreensis*	Actinobacteria							x	x	x		x	x		x	**x**	x	x	x
16	*Streptomyces alkalophilus*													x	x		x	**x**	x	

1, 2, 3, 4 = *h* (m): 0.125, 0.250, 0.375, 0.510 m height.
x: presence of the band in the DGGE profile; in bold bands actually sequenced.

last period of the process, the selected microflora was subjected to a relatively high organic load (24.8 g m^{-3}_{PM} h^{-1}) that induced a sharp increase in species diversity as well as in the quantity of heterotrophic and benzene-degrading bacteria supporting the good biodegradation performance of the biofilter. The increase of the number of species that did not grow on benzene as unique carbon source, could lead to hypothesise that in the bioreactor a complex metabolic network was established, composed by benzene primary degraders and organisms proliferating on degradation by-products and/or on and lysed bacterial cells.

The microbial community was investigated with cultivation-dependent and independent methods, leading the former to describe only the cultivable fraction of the microflora but permitting to gain information on the degrading abilities of the isolates, allowing the latter to theoretically identify all the species present in the biofilter. The results of the two approaches showed a similar picture, permitting to conclude that cultivable bacteria played a major role in benzene degradation under the adopted conditions. In particular, there was a clear correspondence between the molecular signatures of the *Rhodococcus*, *Bordetella* and *Xenophilus* isolates and the fingerprinting patterns of the total population both by ARISA and/or DGGE profiling. Also in the case of *Neisseria*, strain isolation was confirmed on the total DNA of the biofilter by a *Neisseria*-specific PCR only when *Neisseria* was actually isolated, i.e. at VBL = 24.8 g m^{-3}_{PM} h^{-1}. The fact that *Neisseria* was not identified in the DGGE profiles supports the knowledge that biases specific for certain bacteria may affect the efficiency of these methods for their detection. Hence our results demonstrate that for studying the correspondence between cultivable and total bacterial population, the use of multiple molecular methods is of great importance to obtain a reliable picture of the actual community.

The most abundant population with degrading potential was represented by members of the genus

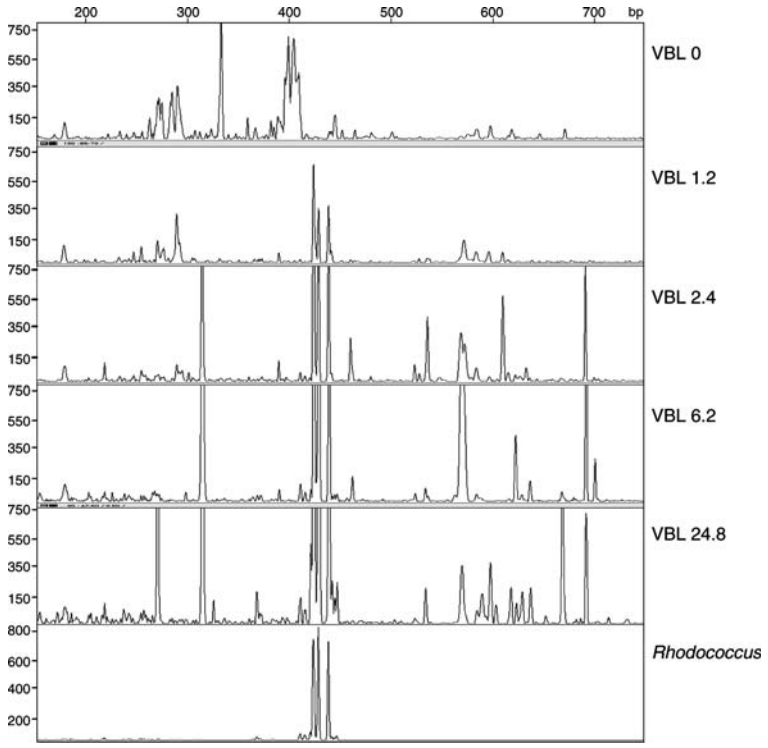

Figure 4. ARISA profiles of compost before the biofilter packing (0) and taken at the central portion of the column ($h = 0.250$ m) using different VBLs (g m^{-3}_{PM} h^{-1}). The ARISA profile at the bottom is that of *Rhodococcus* sp. strain 302.

Rhodococcus, while γ-proteobacteria, among which pseudomonads that are frequently found in biofilters associated to aromatic compounds degradation (Møller et al. 1996; Roy et al. 2003), were in lower amount and only few strains were found to have degrading capability. *Rhodococcus* is not a typical member of the air microflora, but is commonly found in soil matrices and even in the compost (Juteau et al. 1999; Dees & Ghiorse 2001; Namkoong 2004). Hence it was presumably present in the original packing material in a low cell number as compared with the other species, to result under the detection limit of the plate count, DGGE and ARISA methods. The prevalence of *Rhodococcus* cells with degrading capabilities was previously described in a compost-based biofilter for the removal of toluene by Juteau et al. (1999), who hypothesised that this kind of biofilter could constitute a *k*-environment dominated by *k*-strategists genera like *Rhodococcus*, adapted to crowded, resource-restricted environments, which out-competed the *r*-strategists like the pseudomonads, adapted to uncrowded environments rich in nutrients. The biofilter of this study and that used by Juteau et al. (1999), in contrast with the *Pseudomonas*-dominated plant described by Roy et al. (2003), worked for several months before the sampling, resulting in environments depleted of nutrients other than the pollutant, that nevertheless showed an elevated bacterial colonisation, features that would give selective advantages to *k*-strategist.

At the end of the process the biofilter microflora was composed, beside members of the genus *Rhodococcus*, also by α-, β- and γ-proteobacteria and by numerous uncultivable bacteroidetes of the CFB group, similarly to what was found in a previous work on an industrial biofilter treating animal-rendering waste gas (Friedrich et al. 2002). Among the most abundant species found in the biofilter, members of the genera *Bordetella* and *Neisseria*, which include pathogenic species with animal habitat, could be of particular interest. *B. petrii* was never identified among the microflora

of biofilters, or as a benzene-degrader, but it has been recently isolated from a dechlorinating bioreactor and described as the first member of this genus isolated from environments other than animal body (Von Wintzingerode et al. 2001). To our knowledge, this is the first report of isolation of strains belonging to the genus *Neisseria* from an environmental matrix.

Conclusion

This work showed that during a benzene biofiltration process, complex and highly diverse bacterial community efficiently colonised the compost packing material. Strains presumably present in the original compost in very low number grew at expenses of benzene and prevailed at the end of the process over the initial compost microflora, showing a good benzene removal efficiency.

To describe the microbial successions in the biofilter we applied a multiphasic approach since no universal methods have been identified that could completely unravel the complexity of natural microbial assemblages. The simultaneous use of different methods on the same environment could overcome the limitations that affect cultivation-dependent and independent techniques. All the methods revealed that when the process had the maximum benzene removal efficiency, the biofilter was dominated by benzene degrading *Rhodococcus* strains, in contrast with the general finding that Pseudomonads prevail in such bioremediation systems. The prevalence of *Rhodococcus* in the bacterial community was confirmed by cultivation and by cultivation-independent methods, as it was shown by ARISA profiles of the bacterial community. Our results were in accordance with previous works on similar processes treating toluene (Juteau et al. 1999) or gasoline (Namkoong et al. 2004) contaminated air, enforcing the hypothesis that *Rhodococcus* could have a better fitness in crowded resource-restricted environments.

Besides members of the genus *Rhodococcus*, we isolated several strains belonging to the genera *Neisseria* and *Bordetella*, which comprise pathogenic species. The selection in the biofilter of such strains that should be absent in mature compost material, suggests that the biosafety of municipal waste compost and of compost-based biofilters should be carefully investigated.

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